Indole-3-carbinol and a Novel Derivative (OSU-A9) for Bladder Cancer Treatment

A Senior Honors Thesis

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Abstract

Urinary bladder cancer is the one of the most common cancers affecting the Western world. In addition, bladder cancer is considered one of the most expensive cancers to treat due to the necessary surveillance and treatment of recurrences. There is a need for more effective and less toxic treatment strategies as well as potential preventative options. Recently a novel derivative of indole-3-carbinol has been designed and shown to be significantly more potent at inhibiting cancer *in vitro* than the natural parent compound in liver, prostate and breast models. This derivative, named OSU-A9, shows promise for becoming a new and effective drug for the treatment of bladder cancer. *In vitro* effects of I3C and OSU-A9 were studied in both a non-invasive (RT4) and invasive (UMUC3) bladder cancer cell lines. Treatment with I3C and OSU-A9 exhibited dose and time dependent inhibitory effects on the viability of both superficial (RT4) and the invasive (UMUC3) cell lines. In both RT4 cells and UMUC3 cells, OSU-A9 was greater than 100-fold more potent than I3C. In UMUC3 cells, treatment with OSU-A9 caused a does-dependent change in the percentage of the cell population in G2 phase. To evaluate the *in vivo* efficacy of I3C and OSU-A9, female nude mice were subcutaneously injected with 100,000 UMUC3 cells in the bilateral flanks. Our preliminary *in vivo* data indicates that, compared to controls, animals treated with I3C and OSU-A9, both at 25 mg/kg, show reduced tumor weight by 49% and 26% respectively and reduced tumor volume by 61% and 31% respectively. OSU-A9 exhibits more potent anti-cancer activity than the natural parent compound, I3C, *in vitro* for both superficial and invasive bladder cancer. OSU-A9 and I3C inhibited the progression of tumors in preliminary *in vivo* models of invasive bladder cancer. Further

studies are warranted to investigate the mechanisms by which OSU-A9 exhibits anti-cancer activity and its potential for bladder cancer prevention in pre-clinical models. In summary, both the natural compound, I3C, and its derivative, OSU-A9, show promise for being effective treatments for both superficial and invasive bladder cancer.

Introduction

Urothelial carcinoma is one of the most common cancers in the world, ranking fifth in the Western world (1). Most epithelial tumors are believed to progress along a single pathway; urothelial carcinomas have been found to arise through two separate mechanisms each with considerably different behaviors and prognoses (2). Low-grade non-invasive superficial papillary urothelial carcinomas are thought to arise from simple urothelial hyperplasia. These tumors make up about 80% of diagnosed bladder cancers and are easily treatable through surgery and immunotherapy, with a 5-year survival rate of 90%. However, they have a high rate of recurrence and require multiple surgical resections. The other main type, accounting for about 20% of bladder cancers, is invasive and occurs in patients with no previous history of low-grade papillary tumors. These tumors arise *de novo* or from flat, high-grade carcinoma *in situ* (CIS) lesions. Despite therapy, at least half of patients with these invasive tumors die from metastases within 2 years of diagnosis (3). The five-year survival rate for patients with advanced bladder cancer is only 6% with treatment failing in 95% of patients. Factors that have been linked to urothelial tumors include tobacco smoking, exposure to aromatic amines, drinking water laced with arsenic, chronic infection with *Schistosoma* species, radiation therapy, and therapeutic use of alkylating agents (4).

Diet and food intake has long been hypothesized to have a relationship with cancer risk and prevention. Though many epidemiological studies of fruit and vegetable intake and bladder cancer risk have been conducted, it was not until the Health Professionals Follow-up Study that strong evidence was found between food intake and decreased risk of bladder cancer. Results of this study demonstrated a weak, inverse association between total fruit and vegetable intake and bladder cancer risk, but it was not statistically significant (5). In the breakdown of each individual food group, cruciferous vegetables (except for coleslaw) all showed inverse relations between bladder cancer and intake, however only increased broccoli and cabbage intake showed a statistically significant decrease in risk of bladder cancer.

There have been several studies to identify the phytochemicals found in cruciferous vegetables which convey this cancer prevention activity. One compound discovered, glucobrassicin, has been found to inhibit multiple cancers, including lung and stomach cancers in mice and breast cancer in rat (6). When glucobrassicin is hydrolyzed, it gives rise to a number of indole compounds. Indole-3-carbinol (I3C) is one of these metabolites which has shown anti-carcinogenic activity in breast and ovarian cancers (7, 8). More recent studies have shown that I3C induces growth arrest and apoptosis by targeting several cell cycle signaling pathways, including those mediated by Akt, nuclear factor-κB (NF-κB), Bcl-2, mitogen-activated protein (MAP) kinases, the cyclin-dependent kinase (CDK) inhibitors p21 and p27, and cyclin D1 (9-13).

OSU-A9 is a novel compound developed to exploit the antitumor properties of I3C. It is more stable than the natural compound as it does not form acid-catalyzed condensation products as I3C does (14, 15). Besides improved structural stability, OSU-A9 has shown to have 100 fold higher apoptosis-inducing activity than I3C in prostate, breast, and liver cancers both *in vitro* and *in vivo* with very little toxicity to healthy cells (15-17).

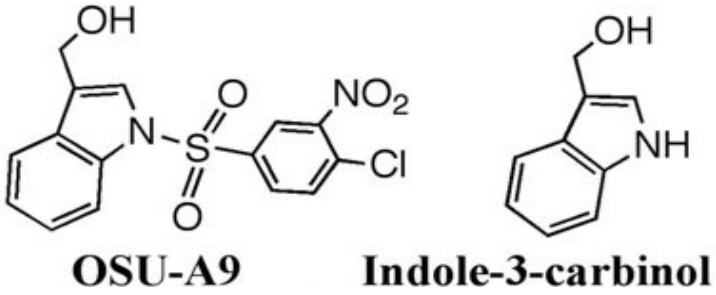


Figure 1. Chemical structures of the natural parent compound I3C and the novel derivative.

In these previous studies, I3C and OSU-A9 have been proven to have antitumorigenic activity and have the potential to become effective treatments for many cancers. Therefore, we aimed to compare the ability of I3C and OSU-A9 to inhibit the growth of superficial and invasive bladder cancer cells *in vitro* and of invasive bladder cancer cells in an *in vivo* xenograft model.

Materials and Methods

Reagents: Indole-3 Carbinol was obtained from Sigma-Aldrich (St. Louis, MO) and OSU-A9 was provided by Dr. Ching-Shih Chen of The Ohio State University College of Pharmacy. I3C and OSU-A9 were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) prior to use.

Cell Culture: RT4 and UMUC3 bladder cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained as monolayer cultures in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2mM L-glutamine, and antibiotic-antimycotic (GIBCO, Grand Island, NY) at 37 °C in a 5% CO_2 / 95% air, humidified atmosphere. Cells were seeded, grown for 24h and then treated with I3C, OSU-A9 or control for 24, 48 or 72 h as reported for each experiment.

Cell Viability: Cells were seeded in 96-well plates, grown for 24h, and treated with culture medium containing I3C, OSU-A9, or vehicle in 0.1% DMSO for 24, 48 or 72h. Cell viability was measured by the SRB assay (In Vitro Toxicology Assay Kit, Sigma-Aldrich, St. Louis, MO) using a HTS 7000 BioAssay reader (Perkin Elmer, Norwalk, CT) at an absorbance wavelength of 565 nm.

Cell Cycle: Cells were seeded in plates, grown for 24h and treated with culture medium containing I3C, OSU-A9, or vehicle, for 24 and 48 h. Cultures were harvested and fixed in 70% ethanol, -20^{0}C, for at least 2 h. Cells were then treated with PBS containing, 0.1% Triton -X100, Propidium Iodide (50 µg/ml), RNase A (200 µg / ml) for 30 min at room temperature. Cell cycle analysis was conducted using the FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA) and data were analyzed with ModFit software (Verity Software House Inc. Topsham, Maine).

In vivo study: Female athymic nude FOXN1nu mice (4-5 week old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were group-housed under alternating 12 hours light and dark cycle, with *ad libitum* access to sterile food and water. Each mouse was injected s.c. with 1 x 10^5 UMUC3 cells, in two flanks, in a total volume of 0.2ml serum free RPMI1640 containing 50% Matrigel (BD Biosciences, Bedford, MA). The animals were then randomly assigned to treatment with I3C (n=3), OSU-A9 (n=5) or control (n=4) groups. Three days after injection, mice began treatment, by daily gavage with either I3C (25 mg/kg body weight), OSU-A9 (25 mg/kg body weight) or vehicle (1% DMSO in soybean oil). Body weights and tumors were measured twice weekly. Tumor volumes were estimated using caliper measurements and using the standard formula: width^2 x length x 0.52 (18). Mice were euthanized before the tumors in any animal reached the maximum allowable tumor burden of a diameter of 1.2 cm. Tumor and organ (bladder, spleen, liver, kidney) weights were measured at necropsy. About half of each tumor and organ was fixed in formalin for histological studies, one quarter snap frozen in liquid N2 for protein analysis, and one quarter stored in RNA*later* Solution (Ambion, Austin, TX) overnight at 4^0C for RNA analysis. All experimental procedures using the mice were in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Statistics: Cell culture-based assays were repeated three times with mean ± SD calculated. Cell lines were examined separately. Outcomes measured at a single time point were analyzed by two-sample t-tests to assess differences between groups.

Differences in xenograft tumor growth were assessed using a two-tailed Student's t-test. Significance was set at $p \leq 0.05$.

Results

OSU-A9 inhibits cell viability of human bladder cancer cells time and dose dependently with higher potency relative to I3C

The effects of indole-3-carbinol and OSU-A9 were evaluated in superficial (RT4) and invasive (UMUC3) human bladder cancer cells. Cells were treated with concentrations of I3C and OSU-A9 from 1-1000 μM or DMSO control for 24, 48 and 72 hours. For each treatment time, there was a dose-dependent inhibition of both cell lines with OSU-A9 being approximately 100X more potent than I3C. After 48 hours of treatment, OSU-A9 had an IC_{50}=2.4 μM and IC_{50}<1 μM for RT4 and UMUC3 cells respectively. The same treatment time with I3C resulted in IC_{50}=280 μM for RT4 cells and IC_{50}=150 μM for UMUC3 cells. Both I3C and OSU-A9 also caused a time-dependent inhibition of viability of RT4 and UMUC3 cells when treated with the same concentration of drug over 24, 48 and 72 hours. Interestingly, the invasive UMUC3 cells were more susceptible to treatment with both compounds with OSU-A9 having a much lower IC_{50} in UMUC3 cells than in RT4 cells. These studies indicate that OSU-A9 and I3C are both

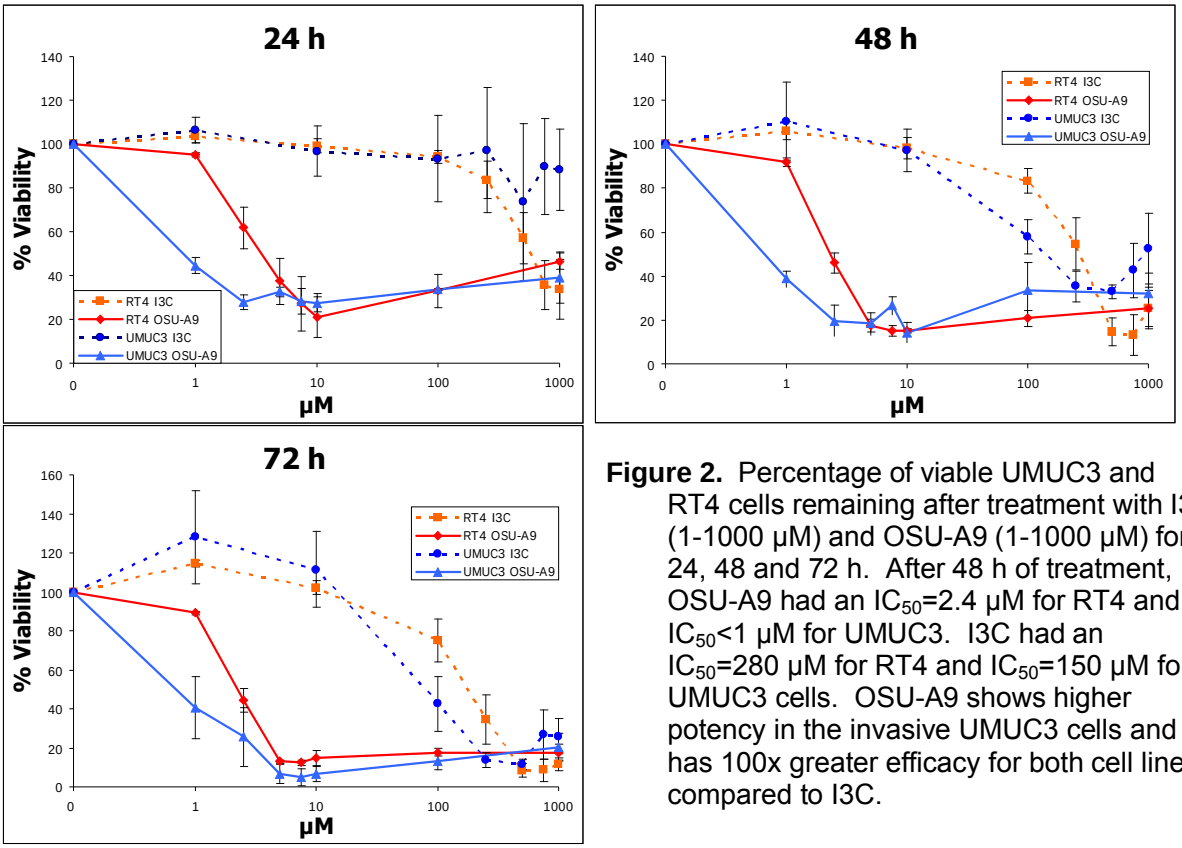


Figure 2. Percentage of viable UMUC3 and RT4 cells remaining after treatment with I3C (1-1000 μM) and OSU-A9 (1-1000 μM) for 24, 48 and 72 h. After 48 h of treatment, OSU-A9 had an IC_{50}=2.4 μM for RT4 and IC_{50}<1 μM for UMUC3. I3C had an IC_{50}=280 μM for RT4 and IC_{50}=150 μM for UMUC3 cells. OSU-A9 shows higher potency in the invasive UMUC3 cells and has 100x greater efficacy for both cell lines compared to I3C.

inhibitors of human bladder cancer cell lines with OSU-A9 having much greater potency and that invasive human urothelial cancer cells are more sensitive to the inhibitory effects of these compounds compared to superficial cancer cells.

Human bladder cancer cells treated with OSU-A9 exhibit markers of cell death and a significant accumulation of UMUC3 cells in G2 phase occurs

In order to determine the effects of these compounds cell cycle progression and apoptosis, RT4 and UMUC3 cells were treated with I3C (200, 400 and 600 μM) and OSU-A9 (1, 5 and 10 μM) or DMSO control for 24 and 48 hours. Cells were fixed and stained with propidium iodide (PI) which intercalates with DNA. The PI staining intensity corresponds to cellular DNA content which is an indication of the phase in the cell cycle. The cell population was viewed by Forward Side Scatter (FSC), a measure of cell size

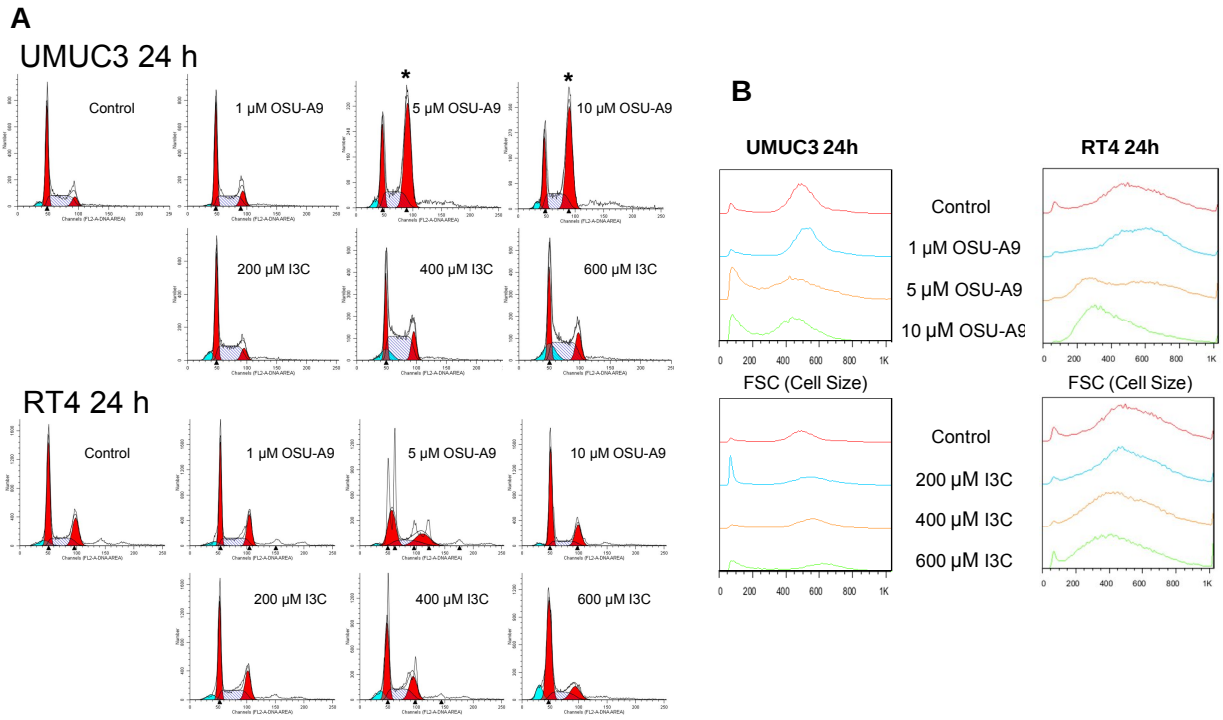
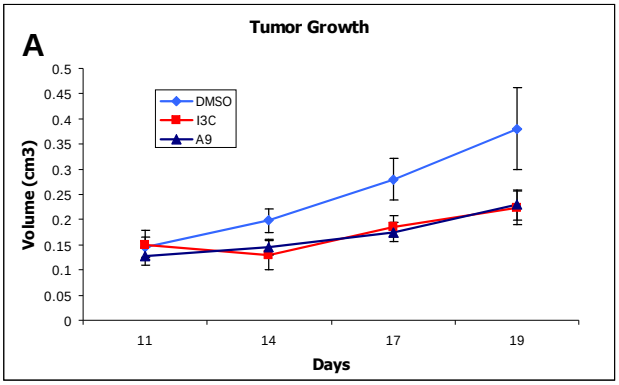


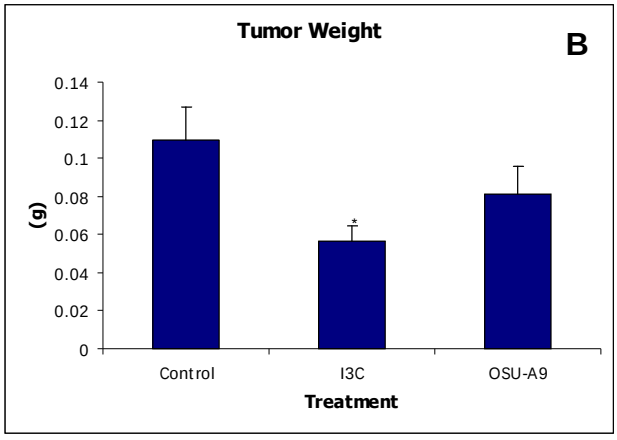
Figure 3. (A) ModFit cell cycle analysis after treatment of 24 h. Treatment of UMUC3 cells with OSU-A9 at 5 and 10 μM causes an accumulation of cells in G2 phase ($p<0.05$). This effect was not observed in UMUC3 cells treated with I3C nor in RT4 cells treated with either OSU-A9 or I3C. Cell debris in the sub-G0/G1 range was not included in order for ModFit to model cell cycle more accurately. (B) Cell size, visualized by Forward Side Scatter (FSC) using FlowJo, decreased in UMUC3 and RT4 cells after treatment with OSU-A9. This is an indication of cell death and mechanisms of apoptosis.

and an indication of cell death. For both UMUC3 and RT4 cells treated with OSU-A9, there was a does-dependent decrease in the size of the cells in the population. This was not seen in cells treated with I3C. Because of the large amount of cell debris in the samples, debris had to be removed by the software before ModFit could accurately model the proportion of cells in each phase of the cell cycle. After removing debris, modeling showed that UMUC3 cells treated with OSU-A9 at 5 and 10 μM for 24 hours and 10 μM for 48 hours had a significant accumulation in G2 phase ($p<0.05$). Cells treated with I3C had no significant changes in their cell cycles.

UMUC3 xenograft tumor growth is significantly inhibited by indole-3-carbinol

We wanted to assess the translation of our *in vitro* findings in an *in vivo* mouse model. We utilized a subcutaneous xenograft tumor model in athymic nude mice, injected with UMUC3 invasive human bladder cancer cells. Starting three days after cells were injected, mice were treated daily by gavage with either I3C (25 mg/kg body weight), OSU-A9 (25 mg/kg body weight) or vehicle (1% DMSO in soybean oil) for 2.5 weeks. Mice were euthanized when tumors reached the maximum diameter of 1.2 cm. There was no evidence of toxicity in any animal treated with I3C or OSU-A9. Animals administered with I3C and OSU-A9 had decreased tumor progression over time compared to animals administered with vehicle. Mean tumor volumes after necropsy for





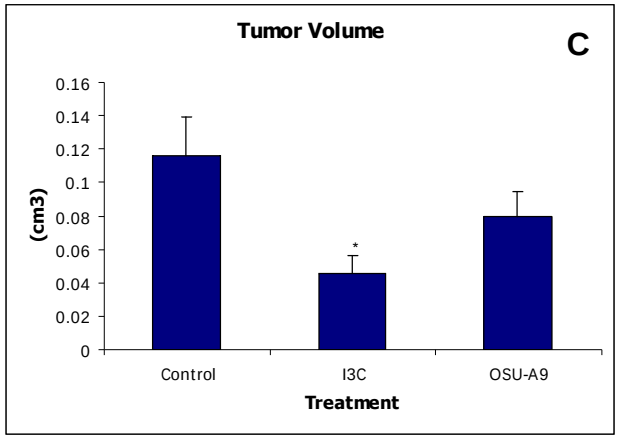


Figure 4. (A) UMUC3 xenograft tumor growth in animals gavaged daily with OSU-A9 (25 mg/kg body weight) and I3C (25 mg/kg body weight) both limited tumor growth compared to tumors on mice treated with DMSO vehicle. (B) Tumor weights measured after necropsy. Both OSU-A9 and I3C inhibited tumor progression compared to DMSO vehicle. I3C was more effective than OSU-A9 (asterisk indicates $p<0.05$). (C) Tumor volumes measured after necropsy. Results are similar to tumor weight (asterisk indicates $p<0.05$). Histopathological evaluation of tumor samples is currently underway.

animals treated with I3C were 61% smaller and tumor weights were 49% less compared to the control mice. While tumor weights and volumes for I3C treated animals were significantly smaller ($p<0.05$), OSU-A9 treated animals also had mean tumor volumes 31% smaller and tumor weights 26% less than control mice tumors (not significant).

Discussion

There is an obvious need for the development of new and effective drugs with increased potency for both bladder cancer treatment and prevention. Nature has already provided numerous compounds and structures which have been proven to have anticarcinogenic properties. By using naturally occurring compounds and novel derivatives based upon their structure, bladder cancer prevention and treatment can be accomplished in pre-clinical models. We show that modifying a natural compound with notable anti-cancer activity *in vitro* can result in new drugs with greatly increased potency. While I3C has a relatively high IC_{50}=280 μM for superficial RT4 bladder cancer cells and IC_{50}=150 μM for invasive UMUC3 cells, the novel derivative greatly reduces cell viability to IC_{50}=2.4 μM and IC_{50}<1 μM for RT4 and UMUC3 cells respectively.

Investigations into the mechanisms of cell death began with the cell cycle studies above. OSU-A9 causes significant cell death in both UMUC3 and RT4 cells and a dramatic shift in the cell size of the population. Also when treated with higher concentrations of OSU-A9, UMUC3 cells have a significant accumulation in G2. Cells treated with I3C did not exhibit any significant changes in cell cycle. It is possible that because both compounds cause such high cell death, they are in fact impacting the cell

cycle at earlier time points. Additional studies are underway to investigate the impact on cell cycle and apoptosis at much earlier treatment times (6 or 12 hours). In addition, we will confirm cell death is occurring through apoptosis using additional analyses including western blotting for caspase 3/7 and other markers of apoptosis. Further western blotting of cell cycle markers involved in cell death and survival such as the NF-kB and Akt pathways will be investigated.

The most surprising results of these studies are the effects of the natural parent compound, I3C, *in vivo*. The *in vitro* viability data indicated that OSU-A9 would also be much more potent than I3C *in vivo*. While OSU-A9 does tend to inhibit UMUC3 xenograft tumor progression, I3C at the same concentration is significantly more bioactive. I3C shows surprising *in vivo* activity suggesting important differences in bioavailability. Tumor samples remain to be analyzed for histopathology and additional biomarkers for the mechanism of action including proliferation and apoptosis. However based upon mean tumor volume and weights I3C is a more effective anti-tumorogenesis agent. I3C shows surprising *in vivo* activity suggesting that metabolic activation may be necessary for bioactivity. In order to better understand this phenomenon, studies on the absorption and metabolism of I3C and OSU-A9 remains to be conducted. This model only tested the chemotherapeutic ability of I3C and OSU-A9 but does not specifically test bladder cancer prevention. A model of carcinogenesis where treatment starts before tumors develop can better test preventative capabilities of these compounds (19, 20).

Taking both the *in vitro* and *in vivo* studies into account, we cannot yet paint a complete picture of the ability of these compounds to treat bladder cancer. It is clear that the novel compound OSU-A9 is capable of greatly increased cell death to human bladder cancer cells *in vitro* but the results of the xenograft study show that we cannot rule out the antitumorogenesis capabilities of the natural parent compound indole-3-carbinol. Our studies have shown that while OSU-A9 could be used as an effective treatment, we must consider I3C as having equal, if not greater potency *in vivo*. Further studies are warranted into both discovering the full potential of the new derivative, OSU-A9, and reinvestigating the abilities of the natural parent, I3C.

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